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Review

Low Lignin Mutants and Reduction of Lignin Content in Grasses for Increased Utilisation of Lignocellulose

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Abstract: Biomass rich in lignocellulose from grasses is a major source for biofuel production and animal feed. However, the presence of lignin in cell walls limits its efficient utilisation such as in its bioconversion to biofuel. Reduction of the lignin content or alteration of its structure in crop plants have been pursued, either by regulating genes encoding enzymes in the lignin biosynthetic pathway using biotechnological techniques or by breeding naturally-occurring low lignin mutant lines. The aim of this review is to provide a summary of these studies, focusing on lignin (monolignol) biosynthesis and composition in grasses and, where possible, the impact on recalcitrance to bioconversion. An overview of transgenic crops of the grass family with regulated gene expression in lignin biosynthesis is presented, including the effect on lignin content and changes in the ratio of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units. Furthermore, a survey is provided of low-lignin mutants in grasses, including cereals in particular, summarising their origin and phenotypic traits together with genetics and the molecular function of the various genes identified.

Keywords: brown midrib; cell wall; gold hull and internode; grass family; lignin; monolignol pathway; mutational breeding; orange lemma; transgenic cereals

1. Introduction

Cereals are a basic food supply for humans and animals worldwide and include rice, maize, wheat, barley and sorghum. They are mainly grown for their nutritional grains that provide dietary calories for human consumption, animal feed and alcoholic beverages. However, whole-crop silage is also a major product in agriculture and is used for animal fodder. Straw from grain production is often considered a by-product, but it is still essential for animal bedding and feed or can be returned to the soil to maintain soil fertility. Additionally, cereals are used in bioindustries for the production of biofuel, textiles, paper, and biochemicals (for a detailed list see [1,2]). The worldwide demand for cereals is growing, but a decrease in their production is starting to be seen [3]. It is therefore crucial to understand the barriers to efficient utility and breeding for new varieties with improved (utility) benefit as feedstuff for animals and bioproducts. In particular, the concept of the multi-purpose crop, in which the grains are used for food and feed and the straw for bioenergy seeks to overcome the food–feed–fuel dilemma by improving the ligno-cellulosic material from straw in second-generation bioethanol production [4].

Lignocellulose is the main component of plant cell walls and the most abundant organic material on earth. It is primarily composed of energy-rich polysaccharides in the form of cellulose, hemicellulose and pectin, rigid phenolic polymers forming lignin and structural (glyco) proteins. The structure is vital for plant growth and serves as a scaffold providing structural and mechanical strength to the plant and protection against external stresses; it encloses each cell individually and facilitates water and solute

flux in the vascular systems [5,6]. Besides these properties, lignocellulose is also an essential source of animal feed and used in various bioindustries [2].

The composition of the lignocellulosic material differs depending on the biomass source, but it usually consists of 20–50% cellulose, 20–30% hemicellulose, 7–30% lignin and 5–35% pectin, with lower amounts of structural proteins that all depend on the plant species, as reviewed by [5,7,8]. Plant cells are made up by two types of cell walls, i.e., primary cell walls (PCW) and secondary cell walls (SCW) placed between the middle lamella and the plasma membrane (Figure 1). PCWs surround all plant cells and are continuously formed during cell growth. The structure is thin and flexible, suitable for elongating cells, but still sufficiently strong to withstand arising turgor pressure [9,10]. It consists primarily of cellulose and hemicellulose, with higher amounts of pectin and proteins in dicots compared to monocots [5,11]. SCWs are formed between the PCW and the plasma membrane in specialised cells such as sclerenchyma and xylem vessels after cell elongation has been completed. They are composed of a greater amount of cellulose and hemicellulose than PCW, and pectin is also partly replaced by lignin. These components form a thicker cross-linked matrix than in PCWs. As mentioned above, the function of lignocellulose is to provide mechanical strength to the cells and to facilitate fluid transport. Lignin is the fundamental component for forming that scaffolding structure and its occurrence has also been documented in PCW and the middle lamella [5,6].

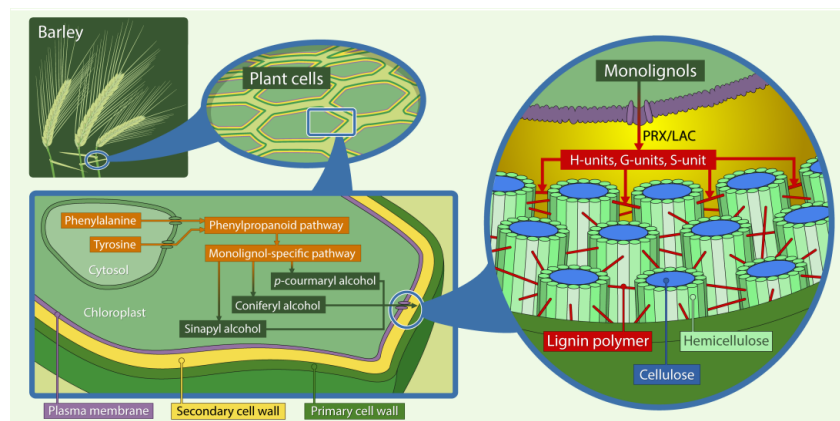


Figure 1. Schematic illustration of the lignocellulosic matrix in the secondary cell wall of the grass family. The main polymers shown are cellulose, hemicellulose, and lignin (shown simplified and not to scale: for microscopic pictures see [12]). They are organised in structures called microfibrils that give structural stability to the plant cell wall. Lignin is the component providing the recalcitrant structure embedding cellulose together with hemicellulose. Lignin is mainly composed of p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, which are derived from 4-hydroxycinnamyl alcohols also known as monolignol, p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The monolignols are synthesised in the cytosol from phenylalanine and tyrosine (grasses only) through the phenylpropanoid pathway and monolignol-specific pathway, then exported across the plasma membranes into the secondary cell wall and oxidized by cell wall-bound peroxidase (PRX) and laccase (LAC), before polymerization into the lignin polymer. (Illustration: Martin Mook).

The recalcitrant structure of lignin is the major limitation of utilising SCWs' nutritional polysaccharides for animal feedstock and producing bioproducts. Lignin also serves as a mechanical defence barrier and is known to accumulate under pathogenic attacks [13–15]. It has also been demonstrated that genes in the monolignol pathway are directly affected by fungal infection [16–18]. For those reasons, lignin biosynthesis has received significant attention, making it one of the most studied pathways [19]. The expression of genes in the pathway has been modified in order to decrease lignin or alter its composition, thus making the pathway a perfect target for precise genome editing [19]. The involvement of transcription factors in lignin biosynthesis has recently been reviewed [20] and will not be discussed further here. Furthermore, both naturally spontaneous and chemically-induced

mutants have been identified and commercialised for animal fodder, showing increased efficiencies for digestion, and are therefore used in breeding programmes. However, in terms of decreasing lignin recalcitrance to bioconversion, there is often a risk of disease infections and dwarfing, depending on the gene being modified [21]. Promising target genes for reduction of lignin recalcitrance without compromising biomass, yield and quality are final genes in the pathway such as *CAD* encoding cinnamyl alcohol dehydrogenase and *COMT* encoding caffeic acid *O*-methyltransferase [22,23]. *CAD* is responsible for reducing cinnamaldehydes to cinnamyl alcohols, the precursors of the building blocks of lignin, also known as monolignols, whereas *COMT* is a multifunctional enzyme, but with a preference for methylations of 5-hydroxyconiferaldehyde to sinapaldehydes and therefore primarily affecting the synthesis of syringyl monolignol [24,25]. The genes responsible for the brown midrib phenotype in (*bm1*, *bm3*) maize and (*bmr6*, *bmr12*) sorghum, which are known for reduced lignin, have mutations within the *CAD* and *COMT* genes affecting their expression. These naturally-occurring low-lignin mutants are of interest for academia and the fodder industry as an alternative source for animal feed and bioproducts [26]. Promoting these well-described varieties avoids the issue of transgenic regulation in Europe, thus increasing the marketing area and also including the organic market. Therefore, downregulating these genes will resemble the naturally-occurring mutants with reduced lignin identified in several cereal crops in the early 20th century.

This review focuses on lignin reduction in important cereals for animal feed (and bioproducts), with a particular focus on papers published after 2010 and updating an earlier review paper, but still including references to primary papers. The aim is (1) to present the monolignol biosynthetic pathway, (2) to provide an overview of recent biotechnology/bioengineering studies targeting genes in the phenylpropanoid and monolignol-specific pathway, and (3) to introduce natural low-lignin mutants with regards to occurrence and phenotypic studies.

2. Lignin Biosynthetic Pathway and Composition in Grasses

Lignin is a phenolic polymer of three units: *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S), which are derivatives of hydroxycinnamyl alcohol, also called monolignols, *p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol, respectively. They only differ in the degree of methylation. The monolignols are synthesised from phenylalanine or tyrosine (exclusively for grasses) [27,28] through the general phenylpropanoid pathway, which is the precursor for numerous specialised metabolites, including flavonoids, tannins and coumarins, and monolignol-specific pathways in the cytosol, before polymerisation in the cell wall. The steps involved in the synthesis are well documented [29,30]. Briefly, phenylalanine and tyrosine are products of the shikimate pathway synthesised in the chloroplasts and exported to the cytosol, where the monolignols are synthesised via a series of enzymatic reactions, illustrated in Figure 2. Deposition of monolignols from the cytosol to the secondary cell wall is unclear, and it is being debated whether they are exported through passive diffusion or actively transported [31]. However, the monolignol-specific pathway is very plastic with numerous inter-specific variations and co-regulated genes. This is explicit with the complex constellation of the lignin polymer, varying in composition between plants and even between cell types. Lignin of grasses primarily consists of S- and G-units. Additionally, grasses also contain H-units and significantly larger amounts of ferulic acid (FA) and *p*-coumaric acid (*p*CA) [11,32]. The FA and *p*CA cross-link to the lignocellulosic matrix, providing structural integrity of the cell wall. They form covalent linkages or ether bonds between polysaccharide and lignin components [33]. Furthermore tricetin, a member of the flavonoid family, has recently been discovered in the lignin polymer and designated an initiator of lignin chains [34,35]. Tricetin is also thought to be found almost exclusively in grasses, with a little amount in other monocots and a few traces in alfalfa [36]. Importantly, the composition of the lignin polymer is relevant in terms of recalcitrance to bioconversion after the lignocellulosic material has undergone thermochemical pretreatment followed by enzymatic or acid/alkaline hydrolysis. The monolignols are coupled with recalcitrant C-C and C-O-C (ether) bonds, providing their recalcitrant structure. However, the coupling of monomers differs: H- and

G-units can couple via β -5 (from monomer-monomer and monomer-oligomer reactions) and 5-5 (from oligomer-oligomer reactions) coupling modes with C-C linkages, whereas S-units are linked with β -O-4 which are more easily degraded [37]. A ratio between the monolignols (S/G ratio) is often used as a validation factor to draw conclusions about cell wall degradation ability. Shortly, a high ratio (above 1.0) indicates more S-units than G-units and a low ratio (below 1.0) indicates less S-unit than G-units. It is often stated that a high ratio favours digestibility, and the reason for that is discussed. One hypothesis is that more S-units compared to G-units increases the number of labile β -O-4 bonds and thereby affects enzymatic digestibility positively. On the contrary, increased S-units lead to a more linear structure with uncondensed (high β -O-4) lignin, which provides higher coverage and interaction with the cellulose fibres and thereby lower enzymatic digestibility. Therefore, using the S/G ratio as a validation factor only partially contributes to biomass recalcitrance. Furthermore, *p*CA linkage with S-units via the ether bond and *p*CA is thought to inhibit fermentation due to toxic effect on yeast [38,39]. Similarly, changes in FA compounds using the monolignol ferulate transferase (*FMT*) gene also affect recalcitrance by introducing more easily broken ester bonds [40,41]. Lignin composition and content can be changed with regards to saccharification by regulating genes in the monolignol-specific pathway without compensating for biomass. Therefore lignin has been a target for genetic manipulation for several decades and remains of interest today.

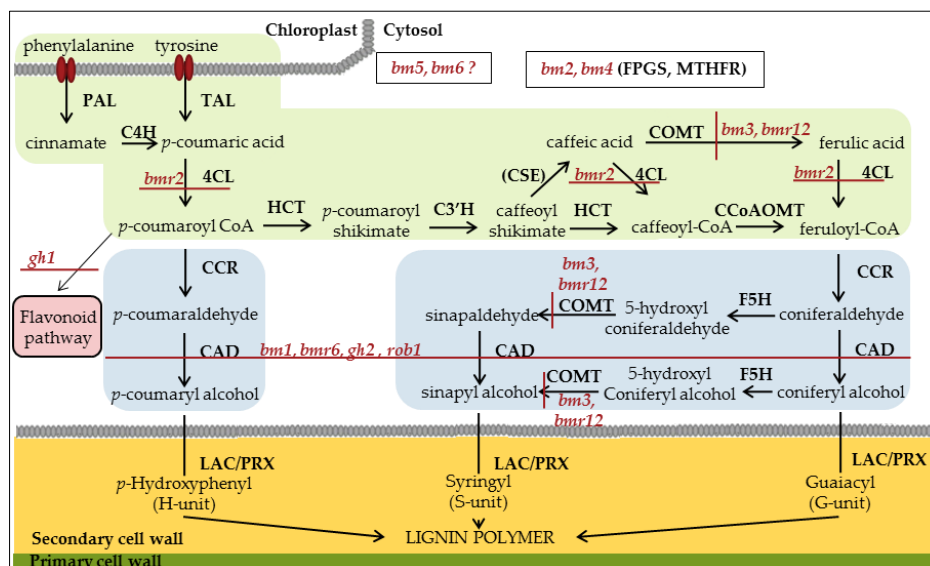


Figure 2. Monolignol biosynthetic pathway for grasses based on studies on *Brachypodium distachyon* [30,32] which is regarded model plant for grasses. The illustration was inspired by [42]. The green box represents the general phenylpropanoid pathway, the blue boxes represent the monolignol-specific pathway, and the light red box indicates *p*-coumarate-CoA as the precursor for the flavonoid pathway. Monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol are synthesised in the cytosol and exported to the secondary cell wall where they undergo oxidation by cell wall-bound peroxidase (PRX) and laccase (LAC) prior to radical coupling in the lignin polymer. Red italic abbreviations for mutants: *brown midrib* maize (*bm*), *brown midrib* sorghum (*bmr*), *orange lemma* barley (*rob*) and *gold hull and internode* rice (*gh*), with identified mutations impairing respective gene enzyme activity indicated by red lines. Enzyme abbreviations: phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), cinnamate 4-hydroxylase (C4H), 4-coumarate coenzyme A ligase (4CL), *p*-hydroxycinnamoyl-CoA:quinic acid/shikimate hydroxycinnamoyl transferase (HCT), *p*-coumarate 3-hydroxylase (C3'H), caffeoyl shikimate esterase (CSE), caffeoyl-CoA O-methyltransferase (CCoAOMT), cinnamoyl CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT) and cinnamyl alcohol dehydrogenase (CAD). Folylpolyglutamate synthase (FPGS) and methylenetetrahydrofolate reductase (MTHFR) are not structural genes in the phenylpropanoid pathway.

3. Biotechnology and Bioengineering of Monolignol Pathway in Grasses

The economic advantages of increasing cereals' nutritional value and replacing fossil fuels with biofuels have driven scientists to investigate and regulate nine of the genes in the monolignol biosynthetic pathway (Table 1), making it an intensively studied pathway. Furthermore, the visual red/orange colouration appearing in stems after downregulating certain genes also makes it attractive as an easy target for new bioengineering methods. The most-used method for regulation and study of the function of genes is “downregulating expression” (of genes) using RNA interference (RNAi). This method introduces small regulatory RNAs (siRNA and miRNA) to the cell, which bind with the RNA-induced silencing complex, Argonaute and other effector proteins, that destroy messenger RNA (mRNA) and thereby prevent the formation of proteins [43]. However, the genes still function and the expression/formation of proteins varies greatly. Repression can be lost completely over a few generations. Furthermore, repression of gene expression does not give a complete picture of the function of a gene, although, it is still a very widely used method. In contrast, using CRISPR/Cas9 to directly knock out gene function by creating stable indel mutations is a more advantageous way of studying gene function [44]. However, in contrast to chemically-induced mutations, CRISPR/Cas9 site-directed mutagenesis requires that the nucleotide sequence of the candidate gene is known before the precise indel mutation can be designed, with stable inheritance over a few generations. This is a relatively new method that has only been used in the most recent studies. However, in July 2018 the EU officially declared that mutations created by CRISPR/Cas9 technology, in contrast to induced mutations, are not exempt from the GMO regulation [45].

It is mostly lignin biosynthetic genes in maize (8) and switchgrass (7) that have been studied by a transgenic approach, with a few in rice (4), Brachypodium (4) and barley (1) (Table 1). Generally, downregulating or knocking out genes leads to a reduced lignin content. However, the estimates of lignin concentration vary greatly depending on the method used for extraction. The most commonly used methods are the gravimetrically determined Klason lignin and the spectrophotometric acetyl bromide lignin method. Briefly, Klason lignin measures insoluble lignin after sulfuric acid hydrolysis of cell walls [46], whereas acetyl bromide lignin is based on the solubility of lignin and measures phenolic compounds' UV absorbance at 280 nm [47]. Large studies have examined and compared quantification methods of lignin and concluded that Klason lignin estimates higher concentrations than acetyl bromide lignin, although both methods are widely used [48,49].

Modifying *PAL*, *4CL* and *C3H* gene expression tends to affect plant growth negatively and induce sterility. However, downregulating genes later in the pathway (*F5H*, *CCoAMT*, *CCR*, *COMT*, and *CAD*) does not have any negative effect on growth (Table 1). This is in contrast with what has been reported for *bm3* mutants, which have mutations in the *COMT* gene [21]. It can be explained by RNAi only reducing gene expression, whereas a complete gene knock out of the candidate gene would have a more drastic effect. The amount of S- and G-units differs greatly between the studies and genes investigated, but there is a general tendency for an overall reduction in S-units. Most studies show that reducing *COMT* gene expression primarily affects the formation of S-units. One study [72] showed that downregulating the *CAD* gene in maize does not result in lignin reduction. This could be due to compensation by other *CAD* genes. Additionally, the expected pigmented phenotype does not appear in any of the grass species when *CAD* is downregulated; it was only observed in *COMT*-downregulated plants. This is in contrast to naturally-occurring low-lignin mutants where both *cad* and *comt* mutants exhibit the pigmented phenotype [26].

Table 1. Transgenic grasses with regulated gene expression in monolignol biosynthesis. The table summarises changes in Klason lignin (KL) or acetyl bromide lignin (ABL) content and changes in the composition of lignin polymer with regards to the amount of syringyl (S), guaiacyl (G) and *p*-hydroxyphenyl (H) units. Notes on other properties affected by gene expression are included, such as a change in growth, resistance, other compounds (mainly ferulic acid (FA) and *p*-coumaric acid (*p*CA)), saccharification based on sugar release, pigmented phenotype and other traits highlighted. The abbreviations for genes are the same as those listed in Figure 1; n.a.: data not available; ↑: increased, ↓: reduced, =: no change compared to wild type.

Gene	Species	Method	Lignin Content	S, G, H	Key Features	References
<i>PAL</i>	Brachypodium	RNAi	↓ 43% (KL)	↑S, ↓G, ↑H	↓growth, ↓pathogenic resistance, ↑saccharification, ↓FA, ↓ <i>p</i> CA	[50]
<i>PTAL1</i>	Brachypodium	RNAi	↓ 43%	↓S, ↑G, ↑H	↓flavone and flavonol derivatives, ↑FA, ↓4CA	[32]
<i>C4H-3</i>	Maize	asRNA	↓ 14–17% (ABL)	n.a.	n.a.	[51]
<i>4CL-1</i>	Switchgrass	CRISPR/Cas9	↓ 8–30% (ABL)	↓S, ↓G, =H	Pigmented phenotype, ↑saccharification, ↑FA, ↑ <i>p</i> CA, linkage bonds changed	[52]
<i>4CL-1</i>	Switchgrass	RNAi	↓22%	=S, ↓G, ↑H	=growth, pigmented phenotype, ↑saccharification	[53]
<i>C3H-1</i>	Maize	RNAi	↓22% (KL)	↓S, ↓G, ↑H	↓growth, sterility, ↑saccharification, ↑anthocyanins, ↑FA, ↑tricin	[54]
<i>C3H</i>	Rice	RNAi	↓30% (KL)	↓S, ↓G, ↑H	=growth, ↑saccharification, ↓FA, ↑ <i>p</i> CA, ↑tricin	[55]
<i>C3H</i>	Rice	CRISPR	n.a.	n.a.	↓growth, ↑death before maturity	[55]
<i>F5H</i>	Brachypodium	Overexpression	↓18% (KL)	↑S, ↓G, ↑H	↑saccharification	[56]
<i>F5H</i>	Rice	RNAi/overexpression	↑/ =	↓S, ↑G, =H/ ↑S, ↓G, =H	=growth, =FA, = <i>p</i> CA/↓growth, ↑sterility, =FA, ↓ <i>p</i> CA	[57]
<i>F5H</i>	Rice	CRISPR	↑25%	↓S, ↑G, =H	=growth, =saccharification, ↑FA, = <i>p</i> CA	[58]
<i>F5H</i>	Sugarcane	RNAi	=	↓S, ↑G	=growth, ↑saccharification	[59]
<i>CCoAOMT-2</i>	Maize	Overexpression	↑	n.a.	↑pathogenic resistance	[60]
<i>CCoAOMT</i>	Maize	RNAi	↓22.4% (KL)	↑S, ↓G	=growth, ↑saccharification	[61]
<i>CCoAOMT</i>	Sugarcane	RNAi	=	n.a.	=growth, ↑saccharification	[59]
<i>CCR-1</i>	Maize	RNAi	↓7–8.7% (KL)	n.a.	<i>bm</i> phenotype, =growth, ↑saccharification	[62]
<i>COMT6 *</i>	Brachypodium	amiRNA	↓24–31.5% (ABL)	↓S, ↓G, =H	Earlier flowering time, ↑saccharification	[63]

Table 1. Cont.

Gene	Species	Method	Lignin Content	S, G, H	Key Features	References
COMT-1,2	Barley	RNAi	↓7–15% (KL)	↓S, ↑G, =H	↑saccharification, ↓pCA, =FA	[64]
COMT	Maize	Antisense downregulation	↓~17%	n.a.	<i>bm</i> phenotype, ↑saccharification	[65]
COMT	Maize	Antisense downregulation	↓25–30% (KL)	↓S, ↑G, ↓H	<i>bm</i> phenotype, ↑saccharification, ↓pCA, =FA	[66]
COMT	Sugarcane	RNAi	=	↓S, ↑G	=growth, ↑saccharification	[59]
COMT	Sugarcane	RNAi	↓4–14% (ABL)	↓S, =G	↓growth, pigmented phenotype, ↑saccharification	[67]
COMT	Sugarcane	RNAi	↓6–12% (ABL)	↓S, ↑G	↓growth, ↑saccharification, =FA, ↓pCA	[68]
COMT	Sugarcane	TALEN	↓29–32% (ABL)	↓S, =G	↓growth, pigmented phenotype, ↑hemicellulose	[69]
COMT	Switchgrass	RNAi	↓11–16%	↓	=growth, ↑saccharification, =pathogenic resistance	[70]
COMT	Switchgrass	RNAi	↓8–9% (ABL)	↓S, =G	=growth, <i>bmr</i> phenotype, ↑saccharification	[71]
COMT	Switchgrass	RNAi	↓11–13% (ABL)	↓S, ↓G	=growth, <i>bmr</i> phenotype, ↑saccharification	[22]
CAD1	Brachypodium	amiRNA	= (ABL)	↓S, ↑G, ↑H	↑growth, delayed flowering, pigmented phenotype, ↑saccharification	[63]
CAD	Maize	RNAi	= (KL)	↓S, ↑G, ↑H	=growth, ↑saccharification	[72]
CAD	Rice	RNAi	n.a.	n.a.	<i>gh</i> phenotype	[73]
CAD	Switchgrass	RNAi	↓14–22% (ABL)	↓S, ↓G	=growth, ↑saccharification, =pCA	[23]
CAD	Switchgrass	RNAi	↓23%	↓S, ↓G	↑saccharification	[74]

* BdCOMT6 (Bradi3g16530) was named BdCOMT4 in the paper [63]. However, based on the accession number and naming in other papers [75,76], BdCOMT6 was chosen. RNAi: RNA interference.

4. Mutants with Reduced Lignin

Naturally-occurring mutants with reduced lignin were identified in cereals such as barley and maize in the early 20th century [77–79]. The mutants are recognised by colour differences: an orange pigmentation occurs in node, lemma and rachis of barley (*rob*) mutants [80], in maize mutants a brown midrib is recognised in the leaves, hence the name ‘brown midrib’ (*bm*) [81], and rice mutants called ‘gold hull and internode’ (*gh*) exhibit a reddish brown pigmentation in the hull and internode [82]. Furthermore, induced mutants with a similar phenotype to *bm* maize have also been identified in sorghum *brown midrib* (*bmr*) mutants and the model plant *Brachypodium* [83,84]. Firstly, *brown midrib* mutants of maize and sorghum were investigated and marketed for ease of forage digestibility [85,86]. With the development of plant molecular biology, the genes responsible for the phenotype have been identified and several biochemical analyses performed [26]. Additionally, low lignin mutants are of great interest in bioethanol production as a replacement for fossil fuel [87]. The sections below give an overview for selected grasses.

4.1. Maize Brown Midrib (*bm*)

Maize (*Zea mays* L.) carrying *bm* mutations are by far the most studied species of all cereals identified with this phenotype. This is because maize silage is an important feed source for dairy cows and other animals. Improving feeding value can affect dairy production and is therefore of high agronomic interest. The first evidence of the positive effect of *bm* mutants on feeding value was

obtained in 1971 [88], and since then analysis has expanded, mostly focusing on the *bm3* mutation. Data concerning feeding efficiency of *bm3* mutants from 1976–2017 have been combined and presented in a newly published review paper by [89]. They conclude that a diet based on *bm3* hybrids has an overall beneficial impact on milk production by dairy cows and reduces the need for energy concentrates. Additionally, knowledge of the impact of other *bm* mutants on cell wall digestibility is still of interest. In total six *bm* mutants have been identified [90,91] and listed in the MaizeGDB database (www.maizegdb.org). A literature search resulted in 191 studies on *bm* mutants, with 60 papers focusing only on *bm3* mutants and just a few on the other mutations. However, some studies investigate several mutants and include double mutants for comparison purposes [17,92,93]. With regards to review papers, previous publications have already discussed identified *bm* mutants and they can roughly be divided into three focus areas: (1) animal feed [89,94–96], (2) bioenergy [97] and (3) biochemical properties and molecular analysis [26,96], with some combining all three subjects [98]. The most recent review published by [89] describes the function of all six *bm* and provides an in-depth analysis of data in relation to animal fodder for *bm3*. However, a short overview of each *bm* is given below.

4.1.1. *bm1*

This *bm* was the first to be identified in maize. The phenotype/trait was discovered by the distinguishable orange/brown midrib in the leaves at three different events [77,99,100] and was described as a simple Mendelian recessive trait. With the discovery of other *bm* loci, it was renamed *bm1*. The *bm1* locus was mapped to chromosome 5 and co-segregates with the *CAD2* (Zm00001d015618) gene [101]. It has been argued that *bm1* only affects the expression of the *CAD2* gene and is not a null mutation. However, it is only recently that *bm1* has finally linked with the *CAD2* gene by sequencing and several different mutations (alleles) in the gene have been identified as being responsible for the phenotype [102]. Phenotypic properties of *bm1* mutants are reduced lignin content, reduced S- and G-units, reduced FA and *p*-CA, increased aldehydes, change in linkage bonds and normal growth as reviewed in [89,102], as well as agronomic properties of increased digestibility and bioethanol.

4.1.2. *bm2*

First described in 1932 by Burnham and Brink [103], *bm2* was mapped fairly recently to the methylenetetrahydrofolate reductase (*MTHFR*, GRMZM2G347056, EC 1.5.1.20) gene at chromosome 1 [81] localised in the cytoplasm [104]. Briefly, *MTHFR* affects methylation of S-adenosyl-L-methionine (SAM) in the methionine cycle, which acts as a methyl donor for CCoAOMT and COMT and thereby the formation of G- and S-units [81,105,106]. Regulating *MTHFR* thus affects the accumulation of both G- and S-units, described by [81]. The *bm2* mutant is caused by a miniature inverted-repeat transposable element (MITEs) insertion, thereby downregulating the function of *MTHFR* [107]. They observed an altered lignin composition in reduced G- (and C-) units, with little change in S-units, which did not affect the total amount of bromide acetyl lignin or growth. It also led to a significant improvement in cell wall saccharification efficiency. Other studies have also observed reduced lignin content and alteration with an increased S:G ratio caused by greatly reduced G-units, a slight increase or unchanged S units and unaffected H-units, reviewed in [26]. Moreover, it has been observed that the *bm2* mutant has the lowest susceptibility to fungus *Ustilago maydis* infection compared to *bm1*, *bm3* and *bm4* mutants [17].

4.1.3. *bm3*

Maize *bm3* was described in 1935 [78] and later linked to chromosome 4, affecting the *COMT* (Zm00001d049541) gene owing to two different mutation events [108,109]. The *bm3* is by far the most studied brown midrib mutant, probably because of its improved feeding values for cattle. It is closely associated with reduced lignin and improved digestion efficiency. The S:G ratio is greatly reduced with *p*-coumarates. Agronomic traits and chemical properties for this mutant have been reviewed very recently [89]. However, there have been no reports on any negative impact associated with *bm3*,

except for one study which shows that the *bm3* mutant has the highest susceptibility for fungal infection when compared to *bm1*, *bm2* and *bm4* mutants [17].

4.1.4. *bm4*

Maize *bm4* was first described by [110] and has been mapped to a putative folylpolyglutamate synthase (*FPGS*, GRMZM2G393334, EC 6.3.2.17) gene at chromosome 9 and expressed in the cytoplasm [104]. *FPGS* catalyses the polyglutamylation of tetrahydrofolate (THF), which subsequently catalyses *bm2*-encoded MTHFR, thus affecting the formation of G- and S-units, similar to *bm2* mutants. The *bm4* mutant is caused by polymorphism in the form of deletions, resulting in a frameshift and premature stop codons. Furthermore, expression analysis indicates that the *bm4* allele is leaky [104]. The effects of *bm2* and *bm4* are correlated [111], however the review by Sattler, Funnell-Harris and Pedersen [26] concludes that they only have modest changes in lignin composition. With regards to biofuel production, a slight increase in glucose release with acid and base pretreatment has been observed for *bm4*, however, the amount is still lower compared to the *bm3* mutant [93]. Moreover, the *bm4* mutant has a reduced defence barrier for pathogenic infection [17].

4.1.5. *bm5*

This natural mutation *bm5* was identified by [112]. It has not yet been linked with a gene, only mapped to chromosome 5 close to *bm1*, but not allelic [113]. There have not been many studies on *bm5*. One study by Mechin, Laluc, Legee, Cezard, Denoue, Barriere and Lapierre [113] observed an increase in H- and S-units with a reduction of G- units, changing the lignin composition, and a reduction in Klason lignin was quantified. Additionally, reduced *pCA* but increased feruloyl esters were linked to the lignin polymer. Finally, it has been suggested that *bm5* is linked to the cinnamoyl CoA reductase gene, based on the incorporation of FA and thereby an increase in the weak *bis* 8-O-4 acetal linkage bonds [113], which can be associated with CCR deficiency [114].

4.1.6. *bm6*

This was first identified by [112] and later mapped to chromosome 2 near bin 2.02 [115]. Only a few analyses have been conducted on *bm6*, but it exhibits reduced height and increased cell wall digestibility [115].

4.1.7. Double Mutants

Several double mutants have been created. They often have adverse growth performances and decreased defence barriers compared to single mutants, however, the rate depends on mutant combination. The defence barrier for fungal infection is substantially reduced for *bm3-bm4*, compared to *bm2-bm3* and single mutants, however *bm2* has a similar infection rate to wild type [17]. In terms of growth performance, double mutants *bm2-bm4* show severely reduced growth and a significantly low maturity rate compared to other double and single mutants, including a reduced lignin content and a reduction in both S- and G-units. In addition, this double mutant also displays a darker brown midrib [111]. Investigations were conducted before *bm2* and *bm4* were linked to a specific gene.

4.2. Barley Orange Lemma (*rob1*)

Barley (*Hordeum vulgare* L.) mutants linked with reduced lignin exhibit an orange colouration in internode, lemma, palea and rachis (Figure 3), hence the locus name “Orange lemma 1” and locus symbol *rob1*. The mutants carrying this phenotype have been identified on several occasions, from both spontaneous and induced mutations (Barley Genetic Newsletter BGS254) [80]. Additionally, germplasm is stored and accessions can be obtained from the U.S. National Plant Germplasm System (<https://www.ars-grin.gov/npgs/index.html>). Even though the *rob1* mutants have been known for almost a century, only a few studies have investigated its utility with regards to animal

feed or biofuel production [116–118]. This is in spite of barley being ranked fourth in cereal production and thus being a major lignocellulosic source. The greatest production is in Europe and Russia, but it is also grown worldwide. It is mainly produced for its nutritional grains for human consumption, animal feed or as malt, with the straw used for animal bedding in rural areas or mostly considered as a waste product [119].



Figure 3. Picture of wild type (WT) barley cv. Golden Promise and barley *rob1* mutant (Rob 13/33) displaying the orange lemma phenotype. (a) Stem, *rob1* shows orange-coloured internodes, (b) spike, *rob1* shows brown rachis and (c) central spikelet, *rob1* show orange/brown palea and lemma close to rachis.

rob1

Rob1 was initially used in inheritance studies and considered to be monofactorial recessive following Mendel with a 3:1 ratio [79,120]. The mutation is located on chromosome 6 near the male-sterile 36 locus and the unicum 2 locus [121] and used as a morphological markers [122–124]. With regards to chemical analysis, one published poster presents the results of *rob1* forage quality, however no differences have been identified between the mutant and the elite cultivars [117], despite measurement of lignin content being 10–15% lower in *rob1* mutants of different backgrounds, as well as altered lignin composition with decreased S:G ratio and increased saccharification efficiency compared to wild type [116,118]. The *rob1* is mapped to the *HvCAD2* gene, similar to *bm1* in maize [116]. However, the detected mutations responsible for the *rob1* mutant have not yet been published.

4.3. Rice Gold Hull and Internode (*gh*)

Rice (*Oryza sativa* L.) displaying the *gold hull and internode (gh)* phenotype has been identified in a number of mutants (*gh1*, *gh2*, *gh3* and *gh4*) listed in the Oryzabase (<https://shigen.nig.ac.jp/rice/oryzabase/>). They are recognised by their reddish-brown pigment in the internode and yellow coloration of the hull. Even though this phenotype was described as early as 1917 [82] and has been used as a marker for a long time [125], it is only recently that a few studies have investigated the genetics behind *gh1* and *gh2* and undertaken biochemical analysis with regards to lignin [82,126,127]. Rice is the second most produced cereal after maize, and it is estimated to be the staple food for one-fifth of the world's population [128]. It is mainly grown in Asia for its grain and its straw is generally used as a waste product. Furthermore, little is used for compost and only a small portion is used for

animal feed, conceivably because the leaves are simply too sharp to be used as animal feed due to their high silicon content. This is a major lignocellulosic source with great potential for utilisation to make various products such as biofuel [129] and byproducts. Therefore, it is suggested that more research on *gh* mutants is needed.

4.3.1. *gh1*

Rice *gh1* is mapped to the chalcone isomerase (*CHI*, Os03g0819600, EC 5.5.1.6) gene on chromosome 3 with a Dasheng retrotransposon inserted causing loss of function [127]. However, this gene is part of the flavonoid pathway, which is derived from the general phenylpropanoid pathway as well as the monolignol pathway [127]. Briefly, the CHI enzyme converts naringenin chalcone, a yellow pigment, into naringenin, and an accumulation of this product causes a yellow pigmentation [126,130]. Since both the flavonoid pathway and the monolignol pathway use the same precursors, one study investigated whether the *gh1* mutant has an effect on lignin formation [126]. Its results showed an increased saccharification efficiency and altered lignin composition with a reduced S:G ratio caused by significantly reduced S-units and increased H- and G-units (and FA). Lignin content differed depending on the extraction method, with reduced thioglycolic lignin content but no change in Klason lignin compared to wild type. Additionally, the *gh1* mutant shows no reduction in biomass or lodging resistance, however reduced grain yield has been reported. This indicates that regulation of genes in the flavonoid pathway affects monolignol formation and lignin composition.

4.3.2. *gh2*

Rice *gh2* phenotype is caused by mutations in the *CAD2* gene (Os02g0187800) on chromosome 2. The original spontaneous *gh2* mutant (Zhefu802) is caused by a point mutation in exon 4 which changes expression level and exhibits the *gh* phenotype [82], while the *gh2* mutant line created with *Tos17* insertion in exon 2 is a null mutant (<https://tos.nias.affrc.go.jp/>) and displays the *bm* phenotype [73]. Expression analysis of the original *gh2* shows reduced CAD and SAD activity differentiating between tissues, which indicates an additional function of CAD-isoenzymes. Klason lignin content is only slightly reduced, even though a dramatic reduction is shown for lignin monomers [82]. Additionally, the *Tos17*-generated *gh2* mutant shows less lignin and increased saccharification efficiency compared to both wild type and spontaneous *gh2* mutant. Furthermore, H- and S-units are also significantly reduced [73]. These two studies indicate the importance of the location of the mutation on the gene. For future research, biomass, grain yield and lodging resistance need to be investigated in order to evaluate the potential of *gh2* as a biofuel crop.

4.4. Sorghum Brown Midrib (*bmr*)

Sorghum (*Sorghum bicolor* (L.) Moench) *brown midrib* (*bmr*) mutants exhibit a similar phenotype to *bm* maize. As the name indicates, a brown coloration in the midrib of leaves is exhibited. The first identified *bmr* mutants were developed via chemical mutagenesis using diethyl sulfate in 1978. Nineteen *bmr* mutants were identified and six mutants (*bmr2*, *bmr6*, *bmr12*, *bmr14*, *bmr18* and *bmr19*) had a significantly reduced lignin compared to wild type [84]. Later, spontaneous *bmr* mutants were also identified by Dr. Gebisa Ejeta (Purdue University, unpublished results) and described in [131,132] and listed consecutively *bmr* 1-28 including the induced *bmr* mutants [87,133,134]. Additionally, a TILLING (Targeting Induced Local Lesions in Genomes) population was examined and even more *bmr* mutants identified [131,135,136]. Allelism tests have been performed and four allelic classes identified—*bmr2*, *bmr6*, *bmr12*, and *bmr19*—with *bmr6* and *bmr12* being the most widely used in breeding programmes [132,137]. *Bmr19* has been reported as having insignificantly reduced lignin and is therefore not of interest to the forage industry [132]. It will therefore not be discussed further in this review. Hence, many *bmr* mutants have been identified and linked to the same locus. In order to obtain a better overview, they have been organised by additional numbers (see [131]). Sorghum is ranked fifth in cereal production. It is mainly distributed in arid areas of Africa, Central America and South Asia,

where it is grown for its grains utilised by humans or as silage for animal feed. Additionally, the stems are used for alcoholic beverages. Considerable research and biochemical analysis have been conducted on *bmr* mutants with regards to both silage and biofuel production. For farmers, the *bmr* phenotype is a visual marker that can be observed in the field to verify the quality trait. A literature search resulted in more than 200 papers published since 1978 when the first *bmr* mutants were developed [84]. Furthermore, many reviews have focused on digestion efficiency, lignin composition and improved saccharification [21,26,86,87,138,139]. Here *bmr2*, *bmr6* and *bmr12* are presented.

4.4.1. *bmr2* Group

Sorghum *bmr2* group, which includes *bmr2*, *bmr5* and *bmr14*, shows a reduction in both G- and S-units, which are all described in [132]. *Bmr2* is the most studied of the three mutants and described as two different alleles *bmr2-ref* [132] and *bmr2-2* [135]. The *bmr2* gene encodes 4CL located on chromosome 4, and sequencing reveals two point mutations within the coding sequence responsible for the phenotype. However, the gene 4CL is part of a family with several isoforms varying in expression regulating different substrates. For a detailed description see [140].

4.4.2. *bmr6* Group

Sorghum *bmr6* group includes *bmr3*, 4, 6, 20, 22–24, 27 and 28 [132]. The *bmr6* phenotype was mapped to the *CAD2* (Sb04g005950) gene on chromosome 4 [141], and different mutations responsible for the *bmr6* phenotype have been revealed by sequencing, resulting in premature STOP-codon or loss of important catalytic domains [141–143]. Reduced *CAD2* activity resulted in decreased lignin content with low amount of G-units and increased level of cinnamaldehydes [144,145]. Another study observed a significant reduction in all lignin subunits, particularly S-units resulting in reduced S:G ratio [142]. In-depth knowledge of the chemical composition, improved saccharification efficiency and decreased lignin content of *bmr6* and *bmr12* question whether the S:G ratio is a valid indicator for lignin recalcitrance and it has been concluded that more knowledge is needed [146]. In terms of agronomic values, lodging is not affected by *bmr6* in either forage [147] or grain sorghum [148], although negative effects on biomass have been reported for forage sorghum [147] and grain yield in grain sorghum [148]. Despite these negative effects, in terms of diets for dairy cows the *bmr6* forage sorghum performs better than wild type [149].

4.4.3. *bmr12* Group

The sorghum *bmr12* group includes *bmr7*, 12, 15, 18, 25 and 26 [132] and are all mapped to the *COMT* gene with premature stop codons giving rise to the *bmr* mutants [150]. Other mutations have also been identified for *bmr12* mutants and characterised by [151]. Overall, the *bmr12* mutants in biomass sorghum all have reduced lignin and generally contribute positively to bioconversion and digestion efficiency [139]. However, negative impacts on agronomical traits have also been reported, such as reduced yield in grain sorghum [148] and biomass in forage sorghum [147], and thus do not affect susceptibility to disease [18]. However, a recent study concludes that weather conditions have a greater impact and in some cases free phenolic compounds even act as a defence mechanism, depending on the diseases reviewed [152].

4.5. Pearl Millet

Pearl millet (*Pennisetum glaucum* L.) is a highly drought-tolerant annual forage plant that is utilised for grain production or as silage for animal feed.

Three brown midrib mutants have been identified in pearl millet, which assembles the same colouration as *bm* maize and *bmr* sorghum. The mutations occurred spontaneously or were induced using dimethyl sulfate. However, only a few studies have investigated the properties of these mutant lines. The agronomic potential is reviewed by [26] and they conclude that a significant yield reduction is associated with *bmr* pearl millets and is therefore not of interest as breeding material.

4.6. *Brachypodium*

Brachypodium distachyon (Brachypodium) is a small grass with a relatively short growing season. It is diploid and the genome is fully sequenced and similar in size to rice. It is therefore used as a model plant for grasses [153].

A chemically-induced mutant population of Brachypodium was developed with a TILLING platform [75]. Several lines were identified with induced mutations in genes involved in the lignin biosynthesis such as *C4H*, *4C*, and *COMT*. The same study analysed the effect of mutations in the *COMT6* (Bradi3g16530) gene on lignin content and composition in several lines. They discovered reduced Klason lignin and altered composition with a decreased S:G ratio, where S-units were significantly reduced and G-units increased. This corresponds with *bm3* and *bmr12* *COMT*-deficient plants [89,151]. Further studies have been performed on line *Bd5139*, which had a missense mutation in the *COMT6* gene, and revealed a reduction in *pCA* esterified to S-units. However, *pCA* linked to arabinoxylans was not affected, which substantiates *comt6* affinity for *pCA* ester-linkage to S-units [76]. Another study also used chemical mutagenesis to create mutations in Brachypodium plants and lignin-deficient mutants were identified by a brownish/red colouration in nodes, lemma and rachis [83]. An SNP mutation was identified in the *CAD1* gene (Bradi3g06480) causing the phenotype; interestingly it was identical to the sorghum *bmr6-3* [141]. Overall the mutant shows reduced lignin and altered composition, which is similar to what has been observed in other species. Furthermore, a coexpression database (www.gene2function.de) has been developed for important genes involved in the lignification of the cell wall in many organs at different developmental stages in Brachypodium [56].

5. Conclusions

Lignocellulosic material from grasses is an essential source for bioethanol production and/or animal fodder. However, the recalcitrant structure of lignin limits decomposition and hence utilisation of the embedded cellulose fibrils. Naturally-occurring low-lignin mutants have been identified in several species and investigations show the great potential in promoting these mutants. So far, however, only *bm* maize and *bmr* sorghum containing mutations have been commercialised. Promoting *gh* rice and *rob1* barley would extend the feedstock source for animals, bioenergy and the emerging circular bioeconomy. Based on existing knowledge about *bm* maize and *bmr* sorghum, it is predicted that there is great potential for improving and developing new commercial varieties of *rob1* barley and *gh* rice with improved utilisation. Furthermore, results from various genetic manipulations of genes in the lignin biosynthesis offers detailed information about the function and its potential for further modification in future research. However, down-regulating genes by antisense/RNAi only provides valid information about gene function and is not useful in breeding. Instead, chemical mutagenesis and CRISPR/Cas9 have the potential to create stable mutations with loss of function, which resembles the natural low-lignin mutants. It has been predicted that CRISPR/Cas9 will revolutionise precision breeding, however there has been a declaration that it now comes under GMO regulations in the EU [45], which complicates the use of this technology. Instead, the screening of existing germplasm is suggested with the use of TILLING to identify new mutations in order to overcome current regulatory difficulties with regard to crop improvements.

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